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PRINCIPAL INVESTIGATOR(S): Kenneth W. Turteltaub

CONTRACTING ORGANIZATION: University of California
Lawrence Livermore National Lab
Livermore, California 94551

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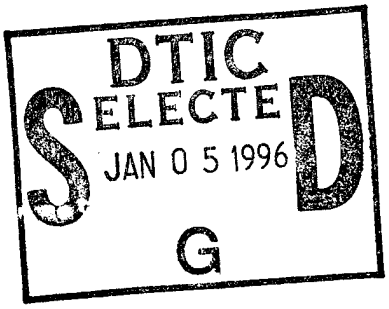

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A great deal of concern has been expressed recently that cooking meat produces genotoxic substances which may contribute to the incidence of human cancers. One important component of these substances is PhIP which has been shown to cause breast tumors in rats. Given the recent findings that mutations in the P53 gene of breast cancer patients are more similar to mutations caused by chemical mutagens than to spontaneous mutations, the role of compounds like PhIP in the etiology of human breast cancer should be critically evaluated. This work is focused on establishing the dosimetry of PhIP in females rodents relative to male rodents at human-relevant exposures. It is also focused on assessing the dose effects of metabolism. Finally it is focused on development of sensitive methods for the measurement of DNA adducts and metabolites in breast tissue that can be later applied to humans to assess susceptibility.

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FOREWORD

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INTRODUCTION

A great deal of concern has been expressed recently that cooking meat produces genotoxic substances which may contribute to the incidence of human cancers. Of all the substances known to be produced during cooking, the most important may be a class of heterocyclic amines called the imidazoazaarenes (AIA's). These heterocyclic amines are considered to be significant because they are produced at relatively low cooking temperatures such as occur through the grilling, frying, and broiling of red meats, poultry, fish, and grain (1-3). Several of these compounds have also been found in beer and wine and in cigarette smoke condensates (4-6). The AIA's currently identified from cooked foods consist of 19 compounds classified generally as quinolines, quinoxalines, phenylpyridines, and carbolines. All quinoline, quinoxaline and carboline AIAs characterized to date are very potent *Salmonella* mutagens (>100,000 rev/ug). 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), a phenylpyridine, is a relatively weak *Salmonella* AIA heterocyclic amine mutagen (2,000 rev/μg), but is the most potent in Chinese hamster ovary cell (CHO) genotoxicity assays (7-9). Other important food-borne carcinogens, such as aflatoxin B_1 or benzo[a]pyrene, are orders of magnitude less potent in genotoxicity assays than the AIAs (10). Importantly, of the 19 known food-borne AIAs, 10 have been tested for carcinogenicity and all ten have been found to induce tumors in both rats and mice; and in multiple organs (2,11,12). Of the AIA's identified, we considered PhIP to be most important since it is present in the highest concentration in well-done beef (2), has been found in cooked grains, beer, wine, and in cigarette smoke; and, unlike most heterocyclic amines, causes breast tumors in the rat (13). Of equal importance, the human exposure of PhIP has been documented as PhIP has been detected in human urine after consumption of normal diets (14,15). Given the recent findings that mutations in the P53 gene of breast cancer patients are more similar to mutations caused by chemical mutagens than to spontaneous mutations, the role of compounds like PhIP in the etiology of human breast cancer should be critically evaluated (16).

A2. Non-human genotoxicity & metabolism

The mechanism of PhIP's genotoxicity has been most adequately characterized in rodents, but several studies have been carried out in nonhuman primates and human tissue fractions. Understanding these mechanisms is critical to determining if PhIP can cause breast cancer in humans and how to predict an individual's susceptibility. Further, understanding these mechanisms is important since species and tissue specificity in metabolism can ultimately affect the extrapolation of the animal data to humans. PhIP is excreted via the urine and feces, and several stable and unstable DNA- and protein-reactive metabolites have been measured and identified (20-25), although pathways may be dose dependent (26). Pharmacokinetics, metabolism, clastogenicity, and DNA adduct formation have also been measured for PhIP, albeit at exposure levels orders of magnitude greater than found naturally and for tissues other than breast (27-36). Some data have been reported in non-human primates (37-40). The sum of the bioassay data shows conclusively that PhIP is a potent genotoxin and carcinogen. The mutagenicity, and presumably the carcinogenicity, of PhIP results from metabolic activation of the parent heterocyclic amine. This principally results from oxidation of the exocyclic amino group to its corresponding N-hydroxylated derivative (2-N-hydroxyamino-1-methyl-6-phenylimidazo[4,5-b]pyridine) by the cytochromes P450 (26,41,42). The initial oxidation of the PhIP molecule by the cytochromes P450 is followed by one of several conjugations of the exocyclic N-hydroxyl group with acetate, sulfate or other constituents (43-45). Interspecies differences in metabolism have been suggested since rabbit P450IA1 is more active with PhIP than the corresponding P450IA2 whereas human, rat, and mouse P450IA2 is more active than the corresponding P450IA1 (28,46,47). Additionally, N-hydroxy-PhIP is preferentially sulfated in mice (44) and preferentially acetylated in human tissue fractions (Turteltaub et al., unpublished). Such interspecies differences in metabolism may be significant for risk

assessment and needs to be understood prior to assessing PhIP's role in human breast cancer. Likewise, the role of the breast in generating bioactive intermediates needs to be understood to develop markers for susceptibility and to understand what makes the breast a target for chemical agents like PhIP.

The principal detoxification pathway for PhIP in rodents and nonhuman primates involves hydroxylation at the 4'-position of the phenyl ring by the cytochromes P450IA (26,38,46,48). The 4'-hydroxyl moiety is subsequently sulfated or glucuronidated to produce several stable excreted metabolites with 4'-PhIP-sulfate [4'-(2-amino-1-methyl-6-phenylimidazo[4,5'-*b*]pyridine)-sulfate] being the predominate metabolite detected in plasma, bile and urine (37,38,48,49). Also detected and identified in urine, plasma and bile are the 4'-PhIP-O-glucuronide [2-amino-4'-(β-1-glucosiduronyloxy)-1-methyl-6-phenylimidazo[4,5-*b*]pyridine], and 4'-hydroxy-PhIP (38,48). Glucuronidation of the N^2- and N^3-positions of the imidazole ring system of the N-hydroxylated PhIP molecule [2-(N-β-1-glucosiduronyl)-2-hydroxyamino-1-methyl-6-phenylimidazo[4,5'-b]pyridine and 3-(N-β-1-glucosiduronyl)-2-hydroxyamino-1-methyl-6-phenylimidazo[4,5'-*b*]pyridine, respectively have also been reported (38,45). Analysis of feces has shown primarily 4'-hydroxy-PhIP and PhIP to be present (38,48). These metabolites may be useful in comparing metabolism among species and in predicting susceptibility since they can be easily measured in urine, blood, and breast fluids. The utility of using this approach, however, remains to be determined and will be addressed through this proposal.

The N^2-PhIP-O-glucuronide and the N^3-PhIP-O-glucuronide, like the N:O-acetylated PhIP, may be meta-stable transportable PhIP metabolites. Meta-stable metabolites may serve to cause damage in tissues where PhIP metabolism does not occur. Indeed, such meta-stable metabolites have been suggested as transportable forms of other N-hydroxylamines which are liberated following hydrolysis in extrahepatic tissues (50,51). These metabolites may be causal factors for the DNA damage seen in the blood cells of primates and rodents given PhIP and for DNA and protein damage in tissues where PhIP metabolism does not occur (38). Importantly, PhIP's metabolism has primarily been established using liver tissue fractions and male animals. Few data are available on the metabolism of PhIP in breast tissue or on metabolite levels in breast fluids. These data are needed to understand PhIP's mechanism of action in inducing breast tumors and for understanding if breast fluids can be used in molecular epidemiology studies. The data gathered through this project will specifically fill in these data voids such that the role of compounds like PhIP in breast cancer can be better understood and used to predict, on an individual basis, who may be at risk. If such an approach proves feasible, it will help be useful in cancer prevention efforts.

A3. DNA and Protein Damage

Exposure to PhIP results in DNA, and likely protein, adduct formation. However, little is known about the identity and sequence specificities of nucleic acid and protein adducts, and in which tissues these most easily form. In addition, tissue specificity in DNA repair is poorly understood. Macromolecular adduction is important since it indicates the active dose of a chemical reaching its target, and is thought to be the initiating event in chemical carcinogenesis. DNA adduct formation with MeIQx has been shown to be quantitatively, but not qualitatively, affected by metabolic capacity (52). PhIP adduct formation may be similarly affected but has not been investigated. N-(deoxyguanosin-8-yl)-3'-monophosphate adducts of IQ, MeIQx and PhIP have been identified and found *in vivo* (39,53-56). Other PhIP adducts also exist and are likewise due to binding at guanines (57). A deoxyguanosin-N^2-yl-PhIP adduct may exist since deoxyguanosin-N^2-yl-MeIQx and MeIQ adducts have been reported.

While most data on the adducts have been derived from studies in the liver, IQ, PhIP and MeIQx have been shown to form adducts in extrahepatic tissues of the rat (12,31,56). High levels of PhIP adducts have been found in the large intestine, white blood cells, pancreas, and heart, followed by stomach, small intestine, kidney, and liver (12,56,58). Some mutational sequence specificity has been demonstrated for *Salmonella* DNA with IQ and PhIP and both inducing GC deletions in the standard frameshift sensitive and uvrB-deficient strains TA98 and TA1538 (59). Protein binding has also been suggested for PhIP (38,60) but, to date, has only been unambiguously demonstrated for IQ (61). A major limitation of the data described above is that all have been derived from high-dose studies and no studies have been reported in the breast even though PhIP causes breast tumors. Thus, little can be determined about the toxicity, biochemistry, and macromolecular targets of PhIP in the breast at human dietary doses.

A4. Human tumorigenesis, genotoxicity, and metabolism

Inadequate data exist on the metabolism and pathologies of all the AIA's, including PhIP, in humans. Several studies have been conducted which show that increased mutagenic activity and some heterocyclic amines can be detected in the urine of fried-meat eaters and men on normal diets, although metabolite recoveries tend to be poor (1, 62-64). Purified human cytochromes P450 and human tissue fractions have been shown to oxidize the AIAs to mutagenic intermediates *in vitro* (65-69). Specifically, liver fractions are known to form the N-hydroxy-PhIP metabolite (70). Further, purified acetyltransferases from human tissues have been used to show that N-hydroxy-PhIP is probably acetylated by the polymorphic arylamine acetyltransferase (71). The paucity of human data can be partially attributed to technical difficulties in measuring metabolism at the low heterocyclic amine concentrations that people are naturally exposed to, and to the difficulty in obtaining material from human subjects. Such difficulty is often methodological in nature. A major goal of the work proposed here will be the development and validation of methods which will allow detection of molecular effects in easily accessible human tissues, such as breast fluids and blood. Development and validation of such methods are important for comparing animal and human metabolism, assessing inter-individual differences in metabolism and for eventual use in identifying high risk individuals, since individual differences in metabolism represents a potentially important determinant in risk associated with carcinogen exposure (72).

B. Significance of the Proposed Work.

B1. The role of dose, metabolism and macromolecular adduct dosimetry in breast cancer

We suggest that the biologically active carcinogen dose to an organism will dictate the biological outcome of the exposure. Dosage effects on DNA adduct formation is of principal interest because it is believed to be an early initiating event in chemical carcinogenesis. Also, DNA adducts are one form of genetic damage that can be quantitatively measured with high sensitivity prior to development of tumors. Protein adducts are likewise important since protein adducts may lead to chromosomal damage and indicate the bioactive dose of carcinogen reaching the target cells. Metabolism is critical for both adduct formation events since PhIP is a procarcinogen, requiring metabolism to reactive or meta-stable metabolites for the macromolecular binding that leads to mutations and cancer. Thus, the levels of macromolecular adducts may indicate metabolic rates (metabolic capacity) and therefore may be an index of risk. Adduct levels, types and quantitative repair rates affect a compound's ability to induce cancer since carcinogenicity appears to be a function of the site within the genome attacked (DNA adducts lead to mutations causing the activation of proto-oncogenes or inactivation of tumor suppresser genes) (73-75). Essentially nothing is presently known about the effects of low level exposure on adduct formation, adduct persistence, adduct repair, or metabolism in the

breast or any other tissue. PhIP tumorigenicity in rodents has been established using chronic feeding, generally at one or two unnaturally high doses (200 - 500 ppm in the diet) (11,17). A few attempts have been made to measure the effect of dose on *in vivo* cytotoxicity (30,36). Dosage effects on DNA adduct formation have only recently been addressed and only for MeIQx (28,56,76). Several studies on AIA pharmacokinetics have been reported but few valid comparisons can be made, since all have been carried out at different doses, with different AIAs and by different routes of exposure (21,24,27,60). Further, most studies, excepting tumor bioassays, have utilized male animals. No systematic study of the effects of dose on the toxicology of PhIP in the breast or in female animals has been undertaken even though mammary tissue is a target site for PhIP-induced tumors.

B2. DNA adduct dosimetry and low-dose metabolism in breast tissue

It seems crucial to elucidate the structural nature of the PhIP adducts in breast, the adducts persistence in breast tissue, and the level of adducts present in low-dose-exposed females. It is of paramount importance to understand how PhIP is metabolized in the breast relative to other tissues, and how exposure dosage and glandular status of the breast affects these endpoints. It is well known that superlinear, linear or sublinear dose-response relationships can occur in tumorigenicity and adduct formation (77,78); and adduct formation/repair have been shown to be both specific (nonrandom) and influenced by chromatin structure (74,79,80). It has also been reported that the breast has the capacity to metabolize some carcinogens (81). Further, carcinogens and drugs are known to be passed to breast milk and can be biologically available to feeding infants (82-84). Understanding the dose-response for both adduct formation and metabolism will significantly improve our ability to extrapolate from the high dose laboratory animal cancer studies to human dose levels. Further, understanding the influence of dose on metabolism will help determine how best to utilize adducts and metabolites for identifying individual women at risk, and what metabolic factors help target chemicals for the breast.

B3. Metabolic capacity and inter-individual susceptibility

It is also crucial that factors affecting variation in PhIP metabolism and its relation to disease susceptibility be understood. It is increasingly apparent that not all individuals have the same risk for disease. This is based, in part, on polymorphisms in drug metabolism. Large differences in the capacity of human lung to metabolize carcinogens have been reported for benzo[a]pyrene (85,86) suggesting potential variability in human lung cancer susceptibility. Polymorphisms in cytochrome P450 have also been shown to occur and some have been correlated with cancer susceptibility (72,87). Significant inter-individual variation in human N-hydroxylation of aromatic amines (including IQ and MeIQx) have also been reported (88-90). Further, human sulfotransferase and acetyltranferase levels have been shown to vary among individuals (91,92; Lang, personal communication) with fast acetylators having a greater susceptibility towards colorectal cancer than slow acetylators, who have a higher risk of bladder cancer (92,93). Collectively, these data suggest that individual susceptibility may be highly influenced by metabolic capacity, and the need to explore this phenomena in breast cancer is critical. This is particularly the case for a compound like PhIP since it causes mammary tumors and since human exposure is widespread. Breast fluids are an ideal sample source since it is easily obtained and may contain metabolites, nucleoside adducts, and protein adducts enabling estimation of both PhIP detoxification and intoxification rates. No data is presently available on the levels of PhIP metabolites, nucleoside adducts, or protein adducts in breast fluids. Further, few attempts have been made to develop assays for measuring these fractions in human breast. Methods for use in humans and data are needed to validate these endpoints as indicators of PhIP exposure and to evaluate their use in predicting individual cancer risk.

B4. Limitations in measurement sensitivity

One of the main limitations of previous approaches has been the lack of sensitivity of the techniques for adduct and metabolite detection. Few methods can approach quantitatively measuring DNA damage or metabolism at real environmental concentration levels (ng - μg/kg/day), although the ^{32}P-postlabeling assay and the USERIA immunoassay have, in some cases, roughly measured DNA adduct levels in occupationally-exposed workers and smokers; and HPLC/blue cotton extraction and gas chromatography-mass spectrometry have been used to monitor heterocyclic amine levels in human urine and feces (14,64,94). However, quantification remains questionable since those measurements are near the detection limits of the techniques. Increases in biological endpoints such as chromosomal aberrations and micronuclei formation are rarely detectable at low doses due to limitations in the number of cells that can be scored. Metabolism studies, which often rely on isotope-tagged molecules, are limited by high backgrounds and the inefficiency of detecting radioactive decay. Other high-sensitivity methods such as capillary electrophoresis (CE), or microbore HPLC with sensitive detection techniques have seen limited application in the study of PhIP metabolism and adduction.

B5. Limitations in measurement specificity

Specificity is another limitation of previous approaches. Mutation frequency analysis tells nothing of the site of covalent attachment of the adduct and has rarely proven sensitive enough for low-dose studies. Immunoassays are limited by the availability of well-characterized, specific antibodies directed against adducts or metabolites. Fluorescence detection methods are limited to fluorescent molecules or systems where appropriate chromophores can be added. The ^{32}P-postlabeling assay, a thin-layer chromatographic separation technique, does provide some specificity in adduct analysis. Unfortunately, adduct standards are needed to utilize fully the information obtained from the postlabeling assay and these are available for very few compounds. Only one PhIP adduct has been chemically characterized, and the nature of the other PhIP adducts is unknown. Detection by ^{32}P-postlabeling of small amounts of adducts or metabolites in breast fluids, in breast tissue following low dose exposure, or breast feeding infants is thus questionable.

B6. The sensitivity and specificity of accelerator mass spectrometry (AMS) for low-dose research

In this project, we will specifically study PhIP metabolism, DNA adduction, and protein adduction in female animals at PhIP concentrations very close to those occurring naturally in the human diet (ng - μg/kg body weight). We will accomplish this task by measuring the distribution of ^{14}C- and ^{3}H-labeled PhIP molecules among the tissues and macromolecules by AMS in combination with traditional means of biochemical separation. AMS is a newly developed technique that is extremely sensitive and specific for counting atomic nuclei (95). This technique allows measurement of the atoms directly as opposed to decay counting, where the isotope is indirectly detected by measuring the small percentage of nuclei that decay in a given time period. The result is 6-7 orders of magnitude sensitivity improvement for isotope detection by AMS compared to decay counting methods. Since the background for ^{14}C detection by AMS has proven quite low (1 part ^{14}C/10^{12} total carbon) and the absolute detection limit has been shown to be 1 part ^{14}C/10^{15} total carbon, measurement of heterocyclic amine molecules in the tissues, macromolecules, and excreta can be measured well below the attomole level (96,97). Besides the advantages offered by increased sensitivity, reduction in sample mass by 10^3 has been common with AMS as compared to decay counting methods (28,98). AMS has been found by our group to be very compatible with existing biochemical separation techniques such as TLC, HPLC, and electrophoresis (Turteltaub, unpublished). Detection limits using AMS with these purification systems are routinely in the low attomole (10^{-18} moles) range. Use of this technology will allow us to measure molecular damage and metabolism at

heretofore unattainably low levels and to directly assess the relationships between dosage, DNA adducts, protein adducts and metabolism in the breast at human dietary levels of exposure.

II. HYPOTHESIS/PURPOSE & SPECIFIC AIMS

The scope of this proposal is to determine if the dietary breast carcinogen PhIP (2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine) causes macromolecular damage in the breast, and the mechanism by which this damage occurs at human levels of exposure. The proposed work will be carried out in female animals for which, as we point out, few data are available. Our purpose is to define the molecular events leading to the development of PhIP-induced breast tumors, and to assess the likelihood that PhIP exposure at human dietary levels present a human breast cancer risk. A crucial step in risk determination is the estimation of the dose of a reactive carcinogen reaching the critical molecular target. DNA adducts are particularly relevant for this purpose since the adduct, if not repaired, can be considered the initial step in the multistage process of cancer. Protein adducts may likewise be useful since they are indicators of the active carcinogen dose in the tissues. Our goals are to understand the effects of chemical dose (exposure) on adduct formation and metabolism, the types of adducts formed, how adducts are repaired, and the ability of the breast to metabolize PhIP at exposure levels expected to occur via the human diet. This low-dose work will be possible by use of AMS, a highly sensitive and novel technique for tracing ^{14}C-labeled xenobiotics with sensitivity in the zeptomole (10^{-21} moles) range. The data collected through this project will help determine if exogenous factors present in the diet can be linked to breast cancer and how best to extrapolate breast cancer risk from standard high-dose tumor assays. Further, this work will lead to a better understanding of the utility of using adducts or metabolites for identifying women at risk for cancer, either because of exposure to high levels of exogenous compounds or due to metabolism genotype. Finally, the data gathered through this work will be used to develop a sensitive assay for assessing PhIP metabolism, exposure and, potentially, risk in humans. If successful, this work will lead in out years to directly studying the molecular epidemiology of PhIP in human breast samples and to defining the role of compounds like PhIP in the etiology of breast cancer.

Specifically, we proposed to:

1) Explore the pharmacokinetics of PhIP in female rodents at low doses to determine if PhIP or its metabolites are taken up by the breast tissue, and how dose influences PhIP's distribution to the breast and breast fluids. Studies will be conducted at doses that range from the animal cancer bioassay doses (mgs/kg/day) to levels equivalent to human dietary exposure (ng - µg/kg/day). (Grant year 1).

2) Determine if [^{14}C]-PhIP is metabolized by the breast of female rodents using rodent tissue homogenates. We will also assess the levels of metabolites passed into the breast fluids of rodents. Metabolites will be separated using HPLC and CE in combination with AMS. Unknown metabolites will be characterized. (Grant year 1 - 2)

3) Establish if PhIP-DNA and -protein adducts form in female rodents, particularly in the breast, at low dose, and what the kinetics of formation/repair are. Breast fluids will also be analyzed for liberated nucleoside and protein adducts, which will then be characterized. (Grant years 1 - 2)

4) Establish the role of dose on PhIP-induced DNA adducts, protein adducts, and metabolism in the breast of female rodents. Studies will be conducted at doses that range from the animal cancer bioassays (mgs/kg/day) doses to levels equivalent to human dietary exposure (ng - µg/kg/day). (Grant years 2 - 4)

5) Develop a highly sensitive AMS-isotope-labeled immunoassay (AMS-ILIA) to detect unlabeled PhIP, stable metabolites, and PhIP adducts in human breast tissue and fluids. This assay will later be used to assess PhIP exposure, as well as, inter-individual and inter-species differences in metabolism. Sensitivity on the order of 10 attomoles (10^{-18} moles) is expected. (Grant years 1-5)

BODY (PROGRESS TO DATE)

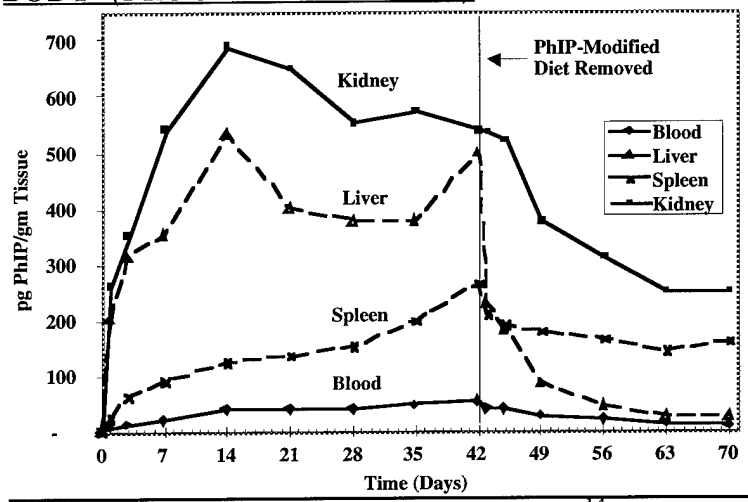

Figure 1 : Accumulation and clearance of ^{14}C-PhIP and PhIP derived products in F344 rat tissues consuming 500 ng PhIP/kg body weight/day for 42 days. At day 42 normal rodent chow was returned.

Work to date has focused on specific aims 1, 2, and 5. Towards meeting the goals of specific aim 1 we have prepared diets containing ^{14}C-PhIP to achieve daily doses of 10-500 ng/kg/day. The goal of this study is to determine the steady-state levels of PhIP available to the tissues and to determine when adduct levels reach equilibrium in the tissues. Animals are being fed the ^{14}C PhIP containing diets for 6 weeks and then are fed a certified rodent chow for an additional 6 weeks. Tissue PhIP and PhIP DNA adduct levels are being measured at selected time points during the 12 wk study. Preliminary analysis of the tissues (Figure 1) from male rats fed a diet modified with PhIP to achieve a dose of 500 ng/kg/day by AMS shows that accumulation of PhIP in some tissues (liver, kidney) increases through 2 weeks and then maintain a steady state while others (blood, spleen) continue to increase through the entire 6 weeks. Steady state levels in the liver average approx. 450 pg PhIP/gm liver while blood levels reach a maximum level of approx. 50 pg PhIP/gm blood at 42 days. After animals were taken off the PhIP-modified diet, adduct clearance varied in the different tissues with rapid clearance from the liver and spleen and slower clearance from kidney and blood. Notably, all tissues measured remain above background levels 4 weeks after removal of the PhIP-modified diet. At 28 after removal of the PhIP-modified chow, the liver retains 26 pg PhIP/gm liver while the spleen (160 pg/gm) and kidneys (250 pg/gm) maintain a higher steady state level of PhIP or PhIP-derived materials. DNA adducts from these tissues are beginning to be analyzed and preliminary data shows a continual increase in liver and colon adduct levels through 35 days. PhiP levels in male brest tissue are also being measured and once completed we will analyze samples from females on the same study regimine. This work will continue into year 2.

Towards specific aim 2 we have conducted a study to determine if PhIP is present in the breast tissue of lactating rats and if PhIP is passed from the milk to suckling pups. Additionally we are developing HPLC/AMS separation protocols for determination of metabolite levels in the milk from these animals. Lactating female F344 rats with suckling pups were gavaged with doses ranging from 50-1000 ng/kg ^{14}C-PhIP. The excretion of the ^{14}C-PhIP in the milk and distribution of ^{14}C-PhIP into the mammary tissue, liver and blood of the dam as well as in the stomach contents, liver and urine of their suckling pups were measured using AMS. ^{14}C-PhIP-derived radioactivity increased in a dose dependent manner in both the milk and stomach contents of the pups as well as in the other tissues measured. Separation of the individual PhIP

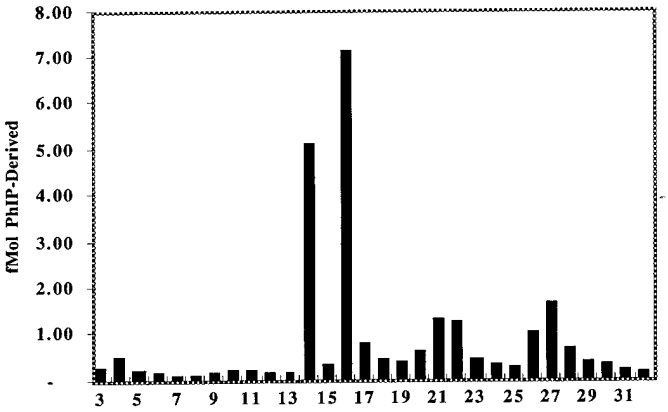

Figure 2 : HPLC/AMS chromatogram of PhIP metabolites found in the milk of a F344 rat dosed with 1000 ng/kg ^{14}C-PhIP.

metabolites by HPLC and analysis of the radiocarbon content of the collected fractions by AMS (Figure 2) indicate 2 major metabolites in the milk (4'-hydroxy PhIP and an unidentified peak). HPLC/AMS analysis of the pup urine are under way. Lactating female rats also were dosed with 500 µg/kg chlorophyllin in conjunction with a 500 µg/kg ^{14}C-PhIP dose. The chlorophyllin treatment caused increased levels of ^{14}C-PhIP in the milk and stomach contents of the pup while decreasing levels in all other tissues measured. HPLC/AMS analysis of both the metabolites found in the milk and pup urine will be analyzed for differences caused by the additional chlorophyllin treatment. The results from these studies suggest that at dietary levels of PhIP, PhIP and/or PhIP metabolites are excreted into the breast milk and absorbed by the newborn. The findings raise the possibility that there is a carcinogenic risk to the newborn by exposure to low levels of PhIP via the breast milk. The addition of chlorophyllin to the dosing regimen demonstrates that other components in the diet may modulate the excretion of ^{14}C-PhIP-derived radioactivity into the breast milk and alter the uptake into tissues of newborns. the effects of addition of chlorophyllin may hvae implications for chemoprevention stradegies. This work is now being prepared for publication.

Towards meeting the goals of specific aim 5 we have conducted several studies to assess the number of potential DNA adducts that PhIP forms with DNA. Our initial study was focused on determining the total number of adducts formed and the optimal conditions for making PhIP DNA adducts with intact DNA. Our goal is to identify which adduct is present in highest yield and to have conditions to make this adduct for use in immunization of rabbits for production of anti-sera. This anti-sera will be used to develop an immunoassay for later use as a marker of exposure in humans. N-hydroxy-PhIP was produced by controlled reduction of nitro-PhIP using palladium/carbon as a catalyst. The resultant N-hydroxy-PhIP was N:O-acetylated by addition of acetic anhydride. The product, N-acetoxy-PhIP, was then added dropwise to solutions of calf thymus DNA, defined sequence oligonucleotides, and deoxyguanosine. For the DNA reactions. The adducted DNA was ethanol precipitated, redissolved in buffer, extensively dialyzed and finally studied using a combination of absorption and fluorescence spectroscopic methods. From these studies 5 DNA adducts were detected. The primary adduct had an absorption max at 395 nm and fluorescence absorption and emission maximums at 365 and 395 nm, respectively. This adduct was tentatively determined to be PhIP-dG-C8. Silver and acrylamide quenching studies showed that this adduct exists in both groove bound and intercalated conformers. The other adducts were not identified in this work. The details are presented in the manuscript published in Chem. Research and Toxicology by Marsch et all (see appendix). Digestion of this DNA and analysis by ^{32}P-postlabeling also suggests 5 adduct peaks (see Mauthe et al., manuscript submitted).

PhIP DNA adducts formed to deoxyguanosine using conditions developed in the study described above are being analyzed by mass spectrometry using a Finnigan TSQ700 mass spectrometer linked via an electrospray interface to microbore HPLC. Initial results show that the primary DNA adduct has an m/z of 490 consistent with the dG-C8 adduct of PhIP. Three additional deoxyguanosine adducts separable by HPLC have mass of 508. Finally an adduct of

mass 532 has been seen. Fragmentation of one of the adducts with mass 508 suggests linkage of the PhIP at the N7 position of deoxyguanosine. The individual adducts are being collected for NMR analysis at the present time.

We have also developed an improved HPLC-based postlabeling assay for use in measuring the individual adducts in animal models. Sensitivity is on the order of 2 adducts/10^9 bases. Postlabeled samples are loaded onto a C_{18} precolumn and adducted bases are retained while excess radioactivity is eluted directly to waste through a switching valve. The use of this inline precolumn enrichment allows entire postlabeled samples to be analyzed without prior removal of excess ATP, inorganic phosphate and unmodified DNA bases. The method has a sample to sample standard error of 15 percent at adduct levels of 2 adduct/10^7 bases and shows a linear relationship between signal and adduction levels down to $\approx 2 \pm 1$ adducts per 10^9 bases. Individual DNA samples (1 to 25 μg) can be analyzed by HPLC in less than 1 hour allowing high throughput of postlabeled samples. Extensions of this technique designed to measure overall adduct levels rather than separate specific PhIP-DNA adducts allows for 15 minute analysis times. These short analysis times allow for more replicates to be measured yielding higher accuracy and precision in the measurements. The use of calf-thymus DNA highly modified with PhIP or DNA isolated from mice chronically fed a PhIP modified diet shows 2 major PhIP-DNA adduct peaks and up to three additional minor adduct peaks when labeled under ATP-limiting conditions. Isolation of the HPLC purified peaks and analysis by thin layer chromatography (TLC) identifies the 2 major peaks as those typically seen by TLC, including the *N*-(deoxyguanosin-8-yl)-2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (dG-C8-PhIP). One of the additional HPLC peaks migrates in the same area as TLC spot 3 while the remaining two HPLC peaks migrate to similar areas as the two major HPLC peaks on the TLC plate. Variations in digestion techniques indicate a potential resistance of the PhIP-DNA adducts to the standard enzymatic digestion methods. Attempts at adduct intensification by solid phase extraction, nuclease P1 enrichment or 1-butanol extraction did not increase PhIP-DNA adduct peaks, but did alter peak ratios and introduced a large early eluting peak. Removal of the 3'-phosphate with nuclease P1 following the kinase labeling reaction simplifies the HPLC profile to one major peak (dG-C8-PhIP monophosphate) with several minor peaks. In addition to the high resolution provided by HPLC separation of the PhIP-DNA adducts, this method can be adjusted for analysis of other DNA adducts and is readily automated for high throughput and decreased handling of ^{32}P. We anticipate application of this assay to measurement of adducts in humans in later years. A manuscript on this study has been prepared and submitted to the journal of Chromatography (see appendix).

CONCLUSIONS

To date, we have been able to make progress on our defined specific aims on several fronts. We have optimized the synthesis for adduct standards needed for the development of antibodies for specific aim 5. We have dosed lactating rats with ^{14}C-PhIP and analyzed tissues of both the dams and pups as well as measured PhIP metabolites in the milk and pup urine in accordance with specific aim 2. We have developed a high throughput ^{32}P-postlabeling assay and a HPLC/AMS capability for support of all specific aims. Finally, we have begun 42 day chronic dosing experiments to examine steady state levels of PhIP in tissues and subsequent clearance rates in accordance with specific aim 1.

We have optimized the conditions for DNA adduct formation as a necessary prerequisite for antibody production and subsequent development of AMS based immunoassays. We have also been able to characterize the major PhIP-DNA adduct (PhIP-C8-dG) and several minor PhIP-DNA adducts by NMR, fluorescence spectroscopy, UV spectroscopy, and mass spectrometry. Using these optimized conditions we will now bw able to begin anti-PhIP antibody production.

We have developed an improved ^{32}P-postlabeling assay to be used for adduct measurements in animal exposures as well as to aid in characterization of the PhIP-DNA adducts. This assay was designed to be flexible to handle a number of different carcinogen-DNA adducts as well as able to determine individual carcinogen-DNA adducts or quickly determine total carcinogen-DNA adduct levels. Analysis times range from 15-60 minutes with sensitivity of approx. 2 adduct/10^9 bases. We have also succeeded in linking HPLC separations with AMS measurements allowing high sensitivity detection of DNA adducts and metabolites from complex matrixes.

We have begun two sets of animal experiments. In the first, we have exposed lactating female rats to PhIP and examined both the dams and pups for PhIP tissue levels. We have also begun to examine the tissues for DNA adducts and the milk and pup urine for metabolite levels. In the same series of experiments, lactating rats were also given a treatment of chlorophyllin in conjunction with PhIP and was found to increase the levels of PhIP excreted through the milk. In a second series of experiments, male F344 rats were administered a diet modified with PhIP to achieve a 10-500 ng/kg/day dose. Tissue and DNA adduct levels have been measured in several tissues. The pharmacokinetics of attaining a steady state of PhIP levels varied dramatically for different tissues. The clearance of the PhIP and PhIP derived materials from the tissues after removal of PhIP from the diet also varied dramatically. Similar studies in female rats will begin soon.

Thus, a great deal of progress has been made in the first year of this project. In the upcoming year we hope to finalize the projects described here as well as begin new phases of the study. We hope to begin immunizing rabbits to collect anti-PhIP antibodies, begin the chronic treatment of female rats and begin using tissue homogenates to examine PhIP metabolism.

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APPENDIX